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RESEARCH ARTICLE

Sex Identification of Four Penguin Species Using Locus-Specific PCR

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Traditional methods for sex identification are not applicable to sexually monomorphic species, leading to difficulties in the management of their breeding programs. To identify sex in sexually monomorphic birds, molecular methods have been established. Two established primer pairs (2550F/2718R and p8/p2) amplify the *CHD1* gene region from both the Z and W chromosomes. Here, we evaluated the use of these primers for sex identification in four sexually monomorphic penguin species: king penguins (*Aptenodytes patagonicus*), rockhopper penguins (*Eudyptes chrysocome*), gentoo penguins (*Pygoscelis papua*), and Magellanic penguins (*Spheniscus magellanicus*). For all species except rockhopper penguins, primer pair 2550F/2718R resulted in two distinct *CHD1Z* and *CHD1W* PCR bands, allowing for sex identification. For rockhopper penguins, only primer pair p8/p2 yielded different *CHD1Z* and *CHD1W* bands, which were faint and similar in size making them difficult to distinguish. As a result, we designed a new primer pair (PL/PR) that efficiently determined the gender of individuals from all four penguin species. Sequencing of the PCR products confirmed that they were from the *CHD1* gene region. Primer pair PL/PR can be evaluated for use in sexing other penguin species, which will be crucial for the management of new penguin breeding programs. Zoo Biol. 32:257–261, 2013.

Keywords: *Aptenodytes patagonicus*; *Eudyptes chrysocome*; *Pygoscelis papua*; *Spheniscus magellanicus*; CHD1

INTRODUCTION

Penguins are globally popular in zoos and aquariums [Andrews et al., 2008; Ghiron et al., 2008; Mann and Mann, 2008; Yamaguchi et al., 2008; Yu et al., 2008]. To maintain penguin colonies at these educational institutions, it is necessary to manage captive breeding programs. However, the success of these programs is limited by correct gender assignment of these sexually monomorphic birds [Bi and Zhao, 2006; Ma and Jia, 2006; Yu et al., 2006; Yu et al., 2008]. In other sexually monomorphic birds, the problem of sex identification is solved in two ways. Traditional methods, such as cloacal examination, biochemical and cytogenetic analysis, and sound discrimination, are time-consuming methods and can stress the animal [Bermúdez-Humarán et al., 2002; Quinn et al., 1990]. Alternatively, a molecular method for sex identification relies on the amplification of the chromo-helicase-DNA-binding 1 (*CHD1*) gene found on the sex chromosomes. Assuming the length of the *CHD1* region differs between the two chromosomes, males will have a single product (*CHD1Z*) and females for two (*CHD1W* and *CHD1Z*) [Ellegren and Sheldon, 1997; Griffiths et al., 1996; Kahn et al., 1998; Sacharczuk et al., 2002] that can be distinguished by gel electrophoresis. In some species, primer pair preferentially amplifies one *CHD1* gene, leading to undetectable amounts of the other one [Fridolfsson and

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Ellegren, 1999; Griffiths et al., 1998]. When this happens, PCR failure of *CHD1Z* fragment results to a detection of only the *CHD1W* gene in females, otherwise, results to the sex failure in females. However, a previous popular study of molecular method focused on birds of Carinatae, while the validity for Impennes were seldom related [Fridolfsson and Ellegren, 1999; Griffiths et al., 1998].

In this study, we evaluate the performance of two established *CHD1* primers in the sex identification of juvenile penguins from four species: king penguins (*Aptenodytes patagonicus,* KP), rockhopper penguins (*Eudyptes chrysocome,* RP), gentoo penguins (*Pygoscelis papua,* GP), and Magellanic penguins (*Spheniscus magellanicus,* MP). Detailed biology information of the four penguin species is shown in Table 1.

MATERIAL AND METHODS

We sampled juvenile penguins from the Hangzhou Polar Ocean Park in China. Our samples included two KPs, two RPs, eight GPs, and 30 MPs. From each individual, we used venipuncture to collect a blood sample. We mixed 1-µl blood with 100-µl anhydrous alcohol and stored the mixture at room temperature. We extracted genomic DNA from the blood samples using a Wizard Genomic DNA Purification Kit (SBS, Shanghai, China) according to the manufacturer's instructions.

We used two established primer pairs, 2550F (5′-GTT ACT GAT TCG TCT ACG AGA-3′)/2718R (5′-ATT GAA ATG ATC CAG TGC TTG-3′) [Fridolfsson and Ellegren, 1999] and p8 (5′-CTC CCA AGG ATG AGR AAY TG-3′)/p2 (5′-TCT GCA TCG CTA AAT CCT TT-3′) [Griffiths et al., 1998], to amplify *CHD1* from the four penguin species. Using Primer3 V0.4.0 [Steve and Helen, 2000] on the sequences amplified by p8/p2, we designed a new primer pair PL (5′-CCC AAG GAT GAT AAA TTG TGC-3′)/PR (5′-CAC TTC CAT TAA AGC TGA TCT GG-3′) to amplify *CHD1* from penguins.

All PCR reactions were conducted on a Techne® TC-5000 (Bibby Scientific, Chelmsford, UK) machine with a final volume of 100 µl containing 1 × PCR buffer (10 mM Tris-Cl, 50 mM KCl, pH 8.3), 0.5 µM of each primer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.25U *Taq* DNA polymerase (Biostar), and 10–100-ng genomic DNA. For all PCRs, an initial denaturation (94°C/5 min) was followed by 36 cycles of denaturation (94°C/30 sec), annealing (2550F/2718R and PL/PR: 55°C/45 sec, p8/p2: 48.5°C/45 sec), and extension (72°C/45 sec), and ended with a final extension (72°C/10 min). Here, we used 100-µl PCR reactions considering the following DNA sequencing, otherwise, 10 µl was enough. All PCR products were separated on 3% agarose gel (Sangon, Shanghai, China), and purified with the DNA Purification Kit (Biostar, Shanghai, China) according to the manufacturer's instructions when sequencing.

Purified PCR products of p8/p2 and PL/PR were cloned into pMD18-T vectors (Takara, Dalian, China) and transformed into DH5α competent cells following the manufacturer's recommendation. After incubation at 37°C on LB

TABLE 1. Detailed biology information for KPs, GPs, RPs, and MPs

Species	Distribution	IUCN[a] criterion	Threats	Mean age at maturation (Y)	Fledging period (days)	References
KP	Subantarctic	LC	Climate change, SST warming	5–6	310–350	Forcada and Trathan, 2009; and references therein
GP	Subantarctic	NT	Climate change, tourism, pollution, fishing, SST warming	3–4	80–105	Forcada and Trathan, 2009; and references therein
MP	Southern coast of South America	NT	climatic change, fishing, marine oil pollution, and garbage	4–5	60–85	Boersma and Stokes, 1995; Petry and Fonseca, 2002; Scolaro, 1987
RP	Subantarctic	VU	Climate change, land predators, ecotourism pollution, fishing, SST warming	≥4	60–70	Forcada and Trathan, 2009; and references therein

KP: king penguin, GP: gentoo penguin, MP: Magellanic penguin, RP: rockhopper penguin, SST: sea surface temperature.
[a]IUCN Red List of Threatened Species (http://www.iucnredlist.org).

agar-ampicillin plates overnight, at least 15 clones per band were checked for an insert by PCR. Between five and 10 insert-positive clones per band were sequenced with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Carlsbad, CA) with the M13 forward primer on an ABI 3730 automated DNA sequencer. Purified PCR products of 2550F/2718R were sequenced directly using the same sequencing system. The sequences were aligned with ClustalX 1.81 [Thompson et al., 1997]. A *CHD1* origin for the PCR products was confirmed by doing a BLAST search against the GenBank nr database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

We used a molecular method for sex identification to determine the sex ratio in captive breeding programs of four penguin species. Using the primer pair 2550F/2718R, we amplified a 665-bp fragment of *CHD1Z* and a 747-bp fragment of *CHD1W* (Fig. 1). When the PCR products were separated by gel electrophoresis, the presence of both bands indicated that an individual was female, while only the shorter band indicated that the individual was male. We amplified two bands from three of the four species (Fig. 2). The amplification of a single band from the two RP samples indicated that either both samples are male or the PCR failed for *CHD1W*. To determine if these results indicate PCR failure, we used primers p8/p2 and amplified a 374-bp fragment of *CHD1Z* and a 392-bp fragment of *CHD1W* (Fig. 3) from all four species. Compared to primers 2550F/2718R, p8/p2 was less powerful for sex identification in penguins. In all cases, the bands were faint and the small size differential between the *CHD1Z* and *CHD1W* fragments made it difficult to distinguish the gender of RPs, GPs, and MPs by 3% agarose gel electrophoresis (Fig. 4).

In addition to fragment size, we distinguished between *CHD1Z* and *CHD1W* by sequencing. We cloned and sequenced the products amplified with 2550F/2718R and p8/p2 (Figs. 1 and 3). The sequences were submitted to GenBank (accession numbers: GU451225-GU451239). For primer pair 2550F/2718R, we detected *CHD1Z* and *CHD1W* fragments from KPs, GPs, and MPs while we only detected *CHD1Z* fragments from RPs. These fragments corresponded to the observed bands (Fig. 2). Both primer pairs gave the same sex identification results for KPs, GPs, and MPs. However, for RPs, we detected only a *CHD1W* fragment from one RP and only a *CHD1Z* fragment from the other one, suggesting that the first RP is female and the second RP is male (Fig. 4). Additionally, these results confirmed that primer pair 2550F/2718R failed for sex identification of RPs. Based on the gel electrophoresis results and sequencing results, we determined the sex ratios (F:M) for juvenile KPs (2:0), RPs (1:1), GPs (3:5), and MPs (17:13).

Because the existing sex identification primers either failed to amplify one of the *CHD1* bands or hard to resolve different bands through 3% agarose gel in a species, we designed a new pair of sex identification primers (PL/PR) from the fragments amplified by primer pair p8/p2. This primer pair amplified distinguishable 276-bp *CHD1Z* and 294-bp *CHD1W* bands for all four penguin species (see in Fig. 3). For KPs, GPs, and MPs, the presence of both bands indicated that an individual was female, while only the shorter band indicated that the individual was male. For RPs, the presence of a shorter band indicated an individual was male while only a longer one for the female (Fig. 5). Sequencing confirmed these results.

DISCUSSION

Right run time can get a clearer image and maximize band differentiation in agarose gel electrophoresis. In this study a time of 50–60 min is recommended with a voltage of 6–8 V/cm.

Primer pairs 2550F/2718R and p8/p2 are powerful tools for the sex determination of most species of birds [Fridolfsson and Ellegren, 1999; Griffiths et al., 1998]. However, our study showed that they did not work for all penguin

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2550F      GTTACTGATTCGTCTACGAGA
MP-CHD1Z   GTTACTGATTCGTCTACGAGAACGTGGCAACAGAGTTCTGATTTTCTCACAGATGGTGAG
RP-CHD1Z   GTTACTGATTCGTCTACGAGAACGTGGCAACAGAGTTCTGATTTTCTCACAGATGGTGAG
GP-CHD1Z   GTTACTGATTCGTCTACGAGAACGTGGCAACAGAGTTCTGATTTTCTCACAGATGGTGAG
KP-CHD1Z   GTTACTGATTCGTCTACGAGAACGTGGCAACAGAGTTCTGATTTTCTCACAGATGGTGAG
KP-CHD1W   GTTACTGATTCGTCTACGAGAACGTGGCAACAGAGTACTGATTTTCTCTCAGATGGTGAG
GP-CHD1W   GTTACTGATTCGTCTACGAGAACGTGGCAACAGAGTACTGATTTTCTCTCAAATGGTGAG
MP-CHD1W   GTTACTGATTCGTCTACGAGAACGTGGCAACAGAGTACTGATTTTCTCTCAGATGGTGAG
           ************************************ *********** ** ********

MP-CHD1Z   GATGCTGGACATCCTAGCAGAATATCTGAAGTATCGTCAGTTTCCCTTTCAGGTAAGAAT
RP-CHD1Z   GATGCTGGACATCCTAGCAGAATATCTGAAGTATCGTCAGTTTCCCTTTCAGGTAAGAAT
GP-CHD1Z   GATGCTGGACATCCTAGCAGAGTATCTGAAGTATCGTCAGTTTCCCTTTCAAGTAAGAAT
KP-CHD1Z   GATGCTGGACATCCTAGCAGAATATCTGAAGTATCGTCAGTTTCCCTTTCAGGTAGGAAT
KP-CHD1W   GATGCTAGACATCCTAGCAGAGTATTTGAAGTATCGTCAGTTTCCCTTTCAGGTAAGAAT
GP-CHD1W   GATGCTAGACATCCTAGCAGAGTATTTGAAGTATCGTCAGTTTCCCTTTCAGGTAAGAAT
MP-CHD1W   GATGCTAGACATCCTAGCAGAGTATTTGAAGTATCGTCAGTTTCCCTTTCAGGTAAGAAT
           ****** ************** *** ************************* *** ****

MP-CHD1Z   CTTGGTGGTAGTAGCCAAGAAGTTTTGATCTTGAATG--------TAAGAAAAATCTT--
RP-CHD1Z   CTTGGTGGTAGTAGCTAAGAAGTTTTGATCTTGAATA--------TAAGAAAAATCTT--
GP-CHD1Z   CTTGGTGGTAGTAGCCAAGAAGTTTTGATCTTGAATA--------TAAGAAAAATCTT--
KP-CHD1Z   CTTGGTGGTAGTAGCCAAGAAGTTTTGATCTTGAATA--------TAAGAAAAATCTT--
KP-CHD1W   TGTGCTGGTAGTAGCCAAGAAGCCTTGATCTTTACCACTTTATCTTAAGAAAAGTGTCCT
GP-CHD1W   TTTGCTGGTAGTAGCCAAGAAGCCTTGATCTTTACCACTTTATCTTAAGAAAAGTGTCCT
MP-CHD1W   TTTGCTGGTAGTAGCCAAGAAGCCTTGATCTTTACCACTTTATCTTAAGAAAAGTGTCCT
             ** ********** ******  ******** *           ******** * *

MP-CHD1Z   -----------------T------------------------------------------
RP-CHD1Z   -----------------T------------------------------------------
GP-CHD1Z   -----------------T------------------------------------------
KP-CHD1Z   -----------------T------------------------------------------
KP-CHD1W   GTTTGTAGAAAGATTTCTTGCGACAGAACAAGGGGGAATGGCTTTAAACTATAGGGGGGT
GP-CHD1W   TTTTGTAGAAAGAT---TTGCGACAGGACAAGGGGGAATGGCTTTAAACTATAGGGGGGT
MP-CHD1W   TTTTGTAGAAAGATTTCTTGTGACAGGACAAGGGGGAATGGCTTTAAACTATAGGGGGGT
                            *

MP-CHD1Z   ----------------------------TCTTTACTCTGAGGGTGGCAGAGCACTGGAAC
RP-CHD1Z   ----------------------------TCTTTACTCTGAGGGTGGCAGAGCACTGAAAC
GP-CHD1Z   ----------------------------TCTTTACTCTGAGGGTGGCAGAGCACTGGAAC
KP-CHD1Z   ----------------------------TCTTTACTCTGAGGGTGGCAGAGCACTGGAAC
KP-CHD1W   AGATCTAGACTAGATACAAGGAAGAAATTTTTTACGCTGAGGGTGGTGAAACACTGGCAC
GP-CHD1W   AGACCTAGACTAGATACAAGGAAGAAATTTTTTACGCTGAGGGTGGTGAAACACTGGCAC
MP-CHD1W   AGATCTAGACTAGATACAAGGAAGAAATTTTTTACACTGAGGGTGGTGAAACACTGGAAC
                                       * ***** **********   * *****  **

MP-CHD1Z   AAGTTGTCCAGAG--GTTATAGAATCTCCATCCTCTGTGACATTGAAAAGCCACCTTGAC
RP-CHD1Z   GAGTTGTCCAGAG--GTTATGGAATCTCCATCCTCTGTGATATTCAAAAGCCACCTTGAC
GP-CHD1Z   AAGTTGTCCAGAG--GTTATGGAATCTCCATCCTCTGTGACATTCAAAAGCCACCTTGAC
KP-CHD1Z   AAGTTGTCCAGAG--GTTATGGAATCTCCATCCTCTGTGACATTCAAAAGCCACCTTGAC
KP-CHD1W   AGGTTGCCCAGAGAGGTGGTGGATGCCCCATCCCTGGAAACATTCAAGGTCAGGTTGGAC
GP-CHD1W   AGGTTGCCCAGAGAGGTGGTGGATGCCCCATCCCTGGAAACATTCAAGGTCAGGTTGGAC
MP-CHD1W   AGGTTGCCCAGAGAGGTGGTGGATGCCACATCCCTGGAAACATTCAAGGTCAGGTTGGAC
             **** ******  **  * **  *  *****   *  * *** **   *    * ***

MP-CHD1Z   ATGACCTTGGGCAACCTGCTTTAGCTG------TCCCTGCCTG-AGTAGGGGAG-TTAGA
RP-CHD1Z   ATGACCTTGGGCAACCTGCTTTAGCTG------TCCCTGCCTG-AGTAGGGGAT-TTAGA
GP-CHD1Z   ATGACCTTGGGGAACCTGATTTACCTG------TCCCTGCCTG-AGTAGGGGAG-TTAGA
KP-CHD1Z   ATGACTTTGGGCAACCTGATTTCACTG------TCCCTGCCTG-AGTAGGGGAG-TTAGA
KP-CHD1W   GGGGCTCTGGGCAACCTGATCTAGTTGAAGATGTCCCTGCCCACAGCAGGGGGG-TTGGA
GP-CHD1W   GGGGCTCTGGGAAACCTGATCTAGTTGAAGATGTCCCTGCTCATTGCAGGGGGGGTTGGA
MP-CHD1W   GGGGCTCTGGGCAACCTGATCTAGTTGAAGATGTCCCTGCTCATTGCAGGGGGG-TTGGA
             * *  **** ****** * *   **      *******     * *****   ** **
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Fig. 1. Alignment of *CHD1Z* and *CHD1W* fragments amplified using 2550F/2718R from four species of penguins. KP = king penguins, RP = rock penguins, GP = gentoo penguins, and MP = Magellanic penguins.

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MP-CHD1Z      CAAGATGACCTCCAGAGGTCCCTTCCAACCTCAACTGTTTTGTGATTATGTCATCTTTAC
RP-CHD1Z      CAAGATGACCTCCAGAGGTCCCTTCCAACCTGAACTGTTTTGTGATTATGTCATCTTTAC
GP-CHD1Z      CAAGATGACCTCCAGAGGTCCCTTCCAACCTCAACTGTTTTGTGATTATGTCATCTTTAC
KP-CHD1Z      CAAGATGACCTCCAGACGTCCCTTCCAACCTCAACTGTTTTGTGATTATGTCATCTTTAC
KP-CHD1W      CTAGATGACCTTTAGAGGTCCCTTCCAACCCAAACTATTCTATGATTCTGTGATTCTA--
GP-CHD1W      CTAGATGACCTTTAGAGGTCCCTTCCAACCCAAACTATTCTATGATTCTGTGATTCTA--
MP-CHD1W      CTAGATGACCTTTAGAGGTCCCTTCCAACCCAAACTATTCTATGATTCTGTGATTCTA--
              * *********  *** *************  **** ** * ***** *** **   *

MP-CHD1Z      CGTTTTGCTTAAGAAAAGATATAAGAAAAAATGTTCTTTTTCTAGAAAGATTGGCAATTG
RP-CHD1Z      CGTTTTGCTTAAGAAAAAATATAAGAAAAAATGTTCTTTTTCTAGAAAGATTGGCAATTA
GP-CHD1Z      CATTTTGCTTAAGAAAAGATATAAGAAAAAGTGTTATTTTTCTAGAAAGATTGGCAATTG
KP-CHD1Z      CATTTTGCTTAAGAAAAGATATAAGAAAAAGTGTTCTTTTTCTAGAAAGATTGGCAATTG
KP-CHD1W      TGATTTATGAAAGTTTAATTTTAAGTACAG--G-----------A-AAAGACTGGTAATTA
GP-CHD1W      TGATTTATGAAAGTTTAATTTTAAGTACAG--G-----------A-AAAGACTGGTAATTA
MP-CHD1W      TGATTTATGAAAGTTTAATTTTAAGTACAG--G-----------A-AAAGACTGGCAAATA
                 ***    ***   *  * **** * *   *          * ***** *** ** *

MP-CHD1Z      CAATATGCTAAATAATATTTTGACATTAAACTGATGAATTAAAAAATTATGTGAAGTGTT
RP-CHD1Z      CAATATGCTAAATAATATTTTGAAATTAAACTGATGAATTAAAAAATTATGTGAAGTGTT
GP-CHD1Z      CAATATGCTAAATAATATTTTGAAATTAAACTGATGAATTAAAAAATTATGTGAAGTGTT
KP-CHD1Z      CGATATGCTAAATAGTATTTTGAAATTAAACTGATGAATTAAAAAATTATGTGAAGTGTT
KP-CHD1W      CTGTATGCTAAATAGTATTTTGAAATGAAACTGATGAATTAGAAAG----ATGAAGTGTT
GP-CHD1W      CTGTATGCTAAATAGTATTTTGAAATGAAACTGATGAATTAGAAAG----ATGAAGTGTT
MP-CHD1W      CTGTATGCTAAATAGTATTTTGAAATGAAACTGATGAATTAGAACG----ATGACGTGTT
              *  *********** ******** ** ************** **       *** *****

MP-CHD1Z      GTAATACTTTTTTTTTCCTTCACATAACAGTTTTGGCAGTTGAGAATTCAAGTTGCTCTG
RP-CHD1Z      GTAATACTTTTTTTT-CCTTCACACAACAGTTTTGGCAGTTGAGAATTCAAGTTGCTCTG
GP-CHD1Z      GTATTACTTTTTTTT-CCTTCACATAACAGTTTTGGCAGCTGAGAATTCAAGTTGCTCTG
KP-CHD1Z      GTATTACTTTTTTGT-CCTTCACATAACAGTTTTGGCAGTTGAGAATTCGAGTTGCTCTG
KP-CHD1W      ACATTACTCTTATTC---CCCCCTCAATTGTTTTGGCAATTGAGAATTCAAGTTGCTCCA
GP-CHD1W      ACATTACTCTTATTC---CCCCCTCAATTGTTTTGGCAATTGAGAATTCAAGTTGCTCCA
MP-CHD1W      GCATTACTCTTATTC---CCCCCTCAATTGTTTTGGCAATTGAGAATTCAAGTTGCTCCG
                * **** ** *       * *  **  *********  ********* ********

MP-CHD1Z      ATTTTGAATATAGTATAAGAATTACTTTTTAACTGTAGTATTCAATCTCTTTAGAGACTT
RP-CHD1Z      ATTTTGAATATAGTATAAGAATTACTTTTTAACTGTAGCATTCAATCTCTTTAGAGACTT
GP-CHD1Z      ATTTTGAATATAGTATAAGAATTACTTTTTAACTGTAGTATTCAATGTCTTTAGAGACTT
KP-CHD1Z      ATTTAGAATATAGTATAAGAATTACTTTTTAACTGTAGTATTCAATCTCTTTAGAGACTT
KP-CHD1W      ATT-AGAATATAGTA--GGAGTTCCTTTTTAACTGTATTATTCAATCTCTTTAGAGACTT
GP-CHD1W      ATT-AGAATATAGTA--GGAGTTCCTTTTTAACTGTATTATTCAATCTCTTTAGAGACTT
MP-CHD1W      ATT-AGAATATAGTA--GGAGTTCCTTTTTAACTGTATTATTCAATCTCTTTAGAGGCTT
              ***  **********   ** ** *************  ******* ********* ***

2718R                                       GTTCGTGACCTAGTAAAGTTA
MP-CHD1Z      GATGGATCAATAAAAGGGGAATTGAGGAAACAAGCACTGGATCATTTCAAT
RP-CHD1Z      GATGGATCAATAAAAGGGGAATTGAGGAAACAAGCACTGGATCATTTCAAT
GP-CHD1Z      GATGGATCAATAAAAGGGGAACTGAGGAAACAAGCACTGGATCATTTCAAT
KP-CHD1Z      GATGGATCAATAAAAGGGGAATTGAGGAAACAAGCACTGGATCATTTCAAT
KP-CHD1W      GATGGATCAATAAAAGGGGAATTGAGGAAACAAGCACTGGATCATTTCAAT
GP-CHD1W      GATGGATCAATAAAAGGGGAATTGAGGAAACAAGCACTGGATCATTTCAAT
MP-CHD1W      GATGGATCAATAAAAGGGGAATTGAGGAAACAAGCACTGGATCATTTCAAT
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Fig. 1. Continued.

species. When we combined our results from both primer pairs, we could identify the sex of individuals from four penguin species. But, the limited resolution between bands amplified by p8/p2 and PCR failures with 2550F/2718R in RPs showed that these primers were not ideal for sex identification in penguins. As a result, we designed primer pair PL/PR, which accurately identified the sex of the four penguin species.

Known limitations of the primer pairs may have led to their failure in penguins. In some species, primer pair 2550F/2718R preferentially amplifies the shorter fragment, leading to undetectable amounts of the longer fragment [Fridolfsson and Ellegren, 1999]. Likewise, for RPs, we observed that 2550F/2718R only amplified the shorter *CHD1Z* and not *CHD1W*. Primer pair p8/p2 is known to

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P8            CTCCCAAGGATGAGRAAYTG
KP-CHD1W      CTCCCAAGGATGATAAATTGTGCAAAACAGGTATCTCTGGGTTTTAACCAACTAACTTTG
RP-CHD1W      CTCCCAAGGATGATAAATTGTGCAAAACAGGTATCTCTGGGTTTTAACCAACTAACTTTG
MP-CHD1W      CTCCCAAGGATGATGAATTGTGCAAAACAGGTATCTCTGGGTTTTAACCAACTAACTTTG
GP-CHD1W      CTCCCAAGGATGATAAATTGTGCAAAACAGGTATCTCTGGGTTTTACCCAACTAACTTTG
RP-CHD1Z      CTCCCAAGGATGATGAATTGTGCAAAACAGGTGTCTCTTGGTT----CTGGCTGACTTGT
MP-CHD1Z      CTCCCAAGGATGATAAATTGTGCAAAACAGGTGTCTCTTGGTT----CTGACTGACTTGT
GP-CHD1Z      CTCCCAAGGATGATGAATTGTGCAAAACAGGTGTCTCTTGGTT----CCGACTGACTTGT
KP-CHD1Z      CTCCCAAGGATGATAAATTGTGCAAAACAGGTGTCTCTTGGTT----CTGACTGACTTGT
PL              CCCAAGGATGATAAATTGTGC
              ************** **************** ***** ****     I*   ** ****

KP-CHD1W      ATTTTTTT--GTTGTTGTTGTT-GTTTGTTTGTTTTTTCGTTGCTGTTGTTTTGGCTTGT
RP-CHD1W      ATTTTTT------GTTGTTGTT-GTTTGTTTGTTTTTTCATTGCTGTTGTTTTGGCTTGT
MP-CHD1W      ATTTTTT------GTTATTGTT-GTTTGTTTGTTTTTTTGTTGCTGTTGTTTTGGCTTGT
GP-CHD1W      ATTTGTTGTTGTTGTTGTTGTTTGTTTGTTTGTTTTTTCGTTGCTGTTGTTTTGGCTTGT
RP-CHD1Z      ACCTTTA-----TGTTGCTGTTGGTTTA---GTTTGGTGGGGGTTGTTGTTGGGTTTTGG
MP-CHD1Z      ACTTTTA-----TGTTGCTGTTGGTTTA---GTTTGTTGGGGGTTGTTGTTGGGTTTTGG
GP-CHD1Z      ACTTTTA-----TGTTGCTGTTGGTTTA---GTTTGTTGGGGGTTGTTGTTGGGTTTTGG
KP-CHD1Z      AC-TTTA-----TGTT------------------------------GTTGTTGGGTGTTGG
              *  * *      ***                               ******  *  ***

KP-CHD1W      ACTTTTGGGTTGGGTGGTTTTCACGCGTGGCGCCCCCTCCCCCCATTTTTGACAGGCTAG
RP-CHD1W      ACTTTTGGGTTGGGTGGTTTTCACGCGTGGCGCCCCCTCCCCCCGTTTTTGACAGGCTAG
MP-CHD1W      ACTTTTGGGTTGGGTGGTTTTCACGCGTGGCGCCCCCTCCCCCCATTTTTGACAGGCTAG
GP-CHD1W      ACTTTTGGGTTGGGTGGTTTTCACGC--GACGCCCCCTCCCCCCATTTTTGACAGGCTAG
RP-CHD1Z      --T--TGGGT----TTTTCTTTTTCC------TTTTCTGAACACATTTTTGACAGGCAAG
MP-CHD1Z      --T--TGGGG----TTTTTTTTTTCC------TTTTCTGAACACATTTTTGACAGGCAAG
GP-CHD1Z      --T--TGGGTGGGTTTTTTTTTTTCC------TTTTCTGAACACATTTTTGACGGGCAAG
KP-CHD1Z      --T--TGGGG----TTTTTTTTTTCC------TTTTCTGAACACATTTTTGACAGGCGAC
                *  ****     *  * **    *          **   * * ******** *** *

KP-CHD1W      ATAACACATTAATAAAATCTTT--GTCACGTAGCTTTGAACTACTTAATCTGAAATTCCA
RP-CHD1W      ATAACACATTAATAAAATCTTT--GTCACATAGCTTTGAACTACTTAATCTGAAATTCCA
MP-CHD1W      ATAACACATTAATAAAATCTTT--GTCACATAGCTTTGAACTACTTAATCTGAAATTCCA
GP-CHD1W      ATAACACATTAATAAAATCTTT--GTCACATAGCTTTGAACTACTTAATCTGAAATTCCA
RP-CHD1Z      GTAAAACTTTACTGATGTTTGTCAGTCACGTAGCTTTGAACTACTTATTCTGAAATTCCA
MP-CHD1Z      GTAAAACTTTACTGATGTTTGTCAGTCGCGTAGCTTTGAACTACTTATTCTGAAATTCCA
GP-CHD1Z      GTAAAACTTTACTGATGTTTGTCAATCACGTAGCTTTGAACTACTTATTCTGAAATTCCA
KP-CHD1Z      GTAAAACTTTACTGATGTTTGTCAATCACATAGCTTTGAACTACTTATTCTGAAATTCCA
PR                                                                     GGT
               *** ** *** * *  * * *   ** * **************** ***********

KP-CHD1W      GATCAGCTTTAATGGAAGTGAAGGGAAACGCAGTAGGAGCAGAAGATATTCTGGATCTGA
RP-CHD1W      GATCAGCTTTAATGGAAGTGAAGGGAAACGCAGTAGGAGCAGAAGATATTCTGGATCTGA
MP-CHD1W      GATCAGCTTTAATGGAAGTGAAGGGAAACGCAGTAGGAGCAGAAGATATTCTGGATCTGA
GP-CHD1W      GATCAGCTTTAATGGAAGTGAAGGGAAACGCAGTAGGAGCAGAAGATATTCTGGATCTGA
RP-CHD1Z      GATCAGCTTTAATGGAAGTGAAGGGAGGCGCAGTAGGAGCAGAAGATACTCTGGATCTGA
MP-CHD1Z      GATCAGCTTTAATGGAAGTGAAGGGAGGCGCAGTAGGAGCAGAAGATACTCTGGATCTGA
GP-CHD1Z      GATCAGCTTTAATGGAAGTGAAGGGAGGCGCAGTAGGAGCAGAAGATACTCGGGATCTGA
KP-CHD1Z      GATCAGCTTTAATGGAAGTGAAGGGAGGCGCAGTAGGAGCAGAAGATACTCTGGATCTGA
PR            CTAGTCGAAATTACCTTCAC
              **************************  ******************** ** ********

KP-CHD1W      TAGTGACTCCATCTCAGAAAGAAAACGACCAAAAAAACGTGGACGACCAC--GAACTATT
RP-CHD1W      TAGTGACTCCATCTCAGAAAGAAAACGACCAAAAAAACGTGGACGACCAC--GAACTATT
MP-CHD1W      TAGTGACTCCATCTCAGAAAGAAAACGACCAAAAAAACGTGGACGACCAC--GAACTATT
GP-CHD1W      TAGTGACTCCATCTCAGAAAGAAAACGACCAAAAAAACGTGGACGACCAC--GAACTATT
RP-CHD1Z      TAGTGACTCCATTTCAGAAAGAAAAAGGCCAAAAAAACGTGGAAGACCAC--GAACTATT
MP-CHD1Z      TAGTGACTCCATTTCAGAAAGAAAAAGGCCAAAAAAACGTGGAAGACCACACGAACTATT
GP-CHD1Z      TAGTGACTCCATCTCAGAAAGAAAAAGGCCAAAAAAACGTGGAAGACCAC--GAACTATT
KP-CHD1Z      TAGTGACTCCATCTCAGAAAGAAA--GGCCAAAAAAACGTGGAAGACCAC--GAACTATT
              ************ ***********  * *************** ******  ********

P2                           TTTCCTAAATCGCTACGTCT
KP-CHD1W      CCTCGAGAAAATATTAAAGGATTTAGCGATGCAGA
RP-CHD1W      CCTCGAGAAAATATTAAAGGATTTAGCGATGCAGA
MP-CHD1W      CCTCGAGAAAATATTAAAGGATTTAGCGATGCAGA
GP-CHD1W      CCTCGAGAAAATATTAAAGGATTTAGCGATGCAGA
RP-CHD1Z      CCTCGAGAAAATATTAAAGGATTTAGCGATGCAGA
MP-CHD1Z      CCTCGAGAAAATATTAAAGGATTTAGCGATGCAGA
GP-CHD1Z      CCTCGAGAAAATATTAAAGGATTTAGCGATGCAGA
KP-CHD1Z      CCTCGAGAAAATATTAAAGGATTTAGCGATGCAGA
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Fig. 3. Alignment of *CHD1Z* and *CHD1W* fragments amplified using p2/p8 and PL/PR from four species of penguins. KP = king penguins, RP = rock penguins, GP = gentoo penguins, and MP = Magellanic penguins.

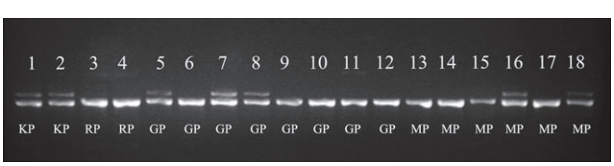

Fig. 2. Gel electrophoresis of *CHD1Z* and *CHD1W* fragments amplified by 2550F/2718R. Males have a single band, while females have two bands, except RPs. Lanes 1 and 2: KPs. Lanes 3 and 4: RPs. Lanes 5–12: GPs. Lanes 13–18: MPs. Not all MP individuals are shown here. KP = king penguins, RP = rock penguins, GP = gentoo penguins, and MP = Magellanic penguins.

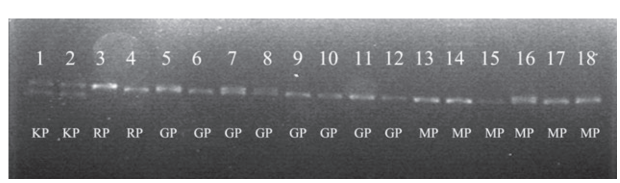

Fig. 4. Gel electrophoresis of *CHD1Z* and *CHD1W* fragments amplified by p8/p2. Lanes 1 and 2: KPs. Lanes 3 and 4: RPs. Lanes 5–12: GPs. Lanes 13–18: MPs. Not all MP individuals are shown here. Lanes 1–18 correspond to the same individuals in Lanes 1–18 in Figure 2. KP = king penguins, RP = rock penguins, GP = gentoo penguins, and MP = Magellanic penguins.

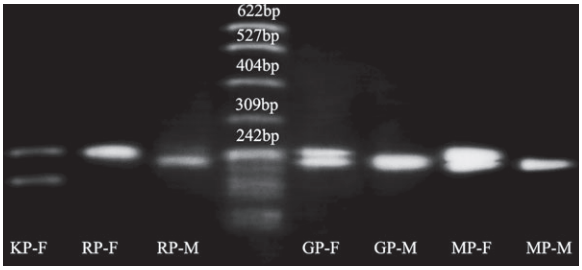

Fig. 5. Gel electrophoresis of *CHD1Z* and *CHD1W* fragments amplified by PL/PR. Lane 1: KP. Lanes 2 and 3: RPs. Lane 4: ladder. Lanes 5 and 6: GPs. Lanes 7 and 8: MPs. KP = king penguins, RP = rock penguins, GP = gentoo penguins, and MP = Magellanic penguins.

exhibit primer competition, in which the primers may match one *CHD1* gene slightly less well than the other [Griffiths et al., 1998]. Here, the p8/p2 results from RPs reflect primer competition. Only primer competition will not result in sexing hindrance. The question is that, as a degenerative primer pair, p8/p2 amplified faint PCR products, the differential size of *CHD1Z/W* fragments made it more difficult to distinguish the PCR products through 3% agarose gel.

Previous attempts of molecular sex identification with 2550F/2718R in MPs were unsuccessful [Bertellotti et al., 2002], but we found that this primer pair successfully distinguished male and female MPs, KPs, and GPs. Other studies investigating sex identification in penguins focused on a single species [Costantini et al., 2008; Poisbleau et al., 2010; Setiawan et al., 2004], limiting their abilities to identify primer pairs that can be used for sex identification in all penguins. We observed that primer pair PL/PR was powerful for molecular sex identification across four penguin species. The introduction of this primer pair should allow for increased sex determination in penguin species.

CONCLUSIONS

1. Primer pair 2550F/2718R can identify the sex of KPs, GPs, and MPs very well, except RPs, through PCR and agarose gel electrophoresis method.
2. Primer pair p8/p2 gives special PCR products for KPs, RPs, GPs, and MPs, of different gender, respectively; while only products of KPs can be clearly separated using agarose gel electrophoresis.
3. The new established primer pair PL/PR can identify the sex of four penguins easily and it is expected to be a valuable primer pair for sexing other penguin species.

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